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14. ABSTRACT

The transmissible spongiform encephalopathies (TSE) chronic wasting disease (CWD) of elk and deer has the potential for transmission to human beings. Reliable antemortem diagnostic tests for CWD are necessary for its control in deer and elk populations. CWD and other TSEs are thought to occur when a normal cellular prion protein (PrPC) mis-folds to an aggregated and degradation resistant PrPSC form. Development of a novel diagnostic probe termed aptamers to detect CWD was proposed. Aptamer selections were conducted against 1) tyrosyl-tyrosyl-arginine (YYR) tripeptide thought to be exposed in PrPsc but not in PrPc, 2) CWD PrPsc, and 3) motif grafted antibodies for two PrP motifs (provided to us by Williams laboratory at the Scripps Research Institute) believed to be involved in mis-folding of PrPC to PrPSC. Selection with the latter two targets resulted in aptamers that recognized PrP as assessed by direct target binding assays. Although not specific for PrPsc, aptamers selected against a grafted motifs for PrP sequences 89-112 bound to CWD PrPsc at higher levels than to rPrPC.

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Introduction

Transmissible spongiform encephalopathies (TSE) such as mad cow disease and its human equivalent variant Creutzfeldt-Jakob disease are transmitted by ingestion of meat contaminated with infective prion protein (PrP^{Sc}). The incubation period for TSEs is many months to years such that it is difficult to identify the source of infection and initiate effective control measures. Recently, a TSE of elk and deer termed chronic wasting disease (CWD) has raised concerns that it too may be transmissible to human beings via ingestion of contaminated meat. Reliable antemortem diagnostic tests for TSEs are necessary for control of these diseases. Because TSEs result from mis-folding of endogenous normal prion protein (PrP^{C}) to form protease resistant PrP^{Sc} isoform, diagnostic tests need to sensitively detect PrP^{Sc} in samples and specifically distinguish PrP^{Sc} from PrP^{C}. Monoclonal antibodies (Mab) have not been produced which are both sensitive and specific for PrP^{Sc}. Commercial Mab-based immunoassays are available for use in detecting PrP^{Sc} in biopsy or postmortem histologic or homogenized brain tissue samples, but these require removal of PrP^{C} by proteinase K digestion. Therefore, a rapid, specific method of direct antemortem identification of PrP^{Sc} is needed. At the time that this project was proposed, novel diagnostic probes termed aptamers have been developed to PrP^{C}, however, these aptamers did not distinguish between PrP^{C} and PrP^{Sc} . Aptamers have an advantage over Mabs because aptamers can be engineered to signal the results of a diagnostic test directly, whereas Mab require extensive protocols to produce results. These engineered aptamers are called aptamer beacons. We are developing aptamer beacons as molecular switches to turn “on” a novel diagnostic technology termed amplifying fluorescing polymer (AFP) when an infectious agent is present. AFP is 1,000 times more sensitive than currently available diagnostic technologies. The proposed project will develop aptamer beacons to CWD for subsequent use in developing an AFP-based antemortem diagnostic test for CWD.

Body

The **Statement of Work** included the Objectives 1 and 2:

Objective 1 Select aptamers for CWD abnormal prion (PrP^{Sc}) (Months 1-15)

Tasks 1.1 Develop protocol for peptide library SELEX (Months 1-3)

1.2 Conduct SELEX with peptide library array for deer prion (PrP_{91-245}) (Months 3-9)

1.3 Prepare and assess preparation of crude cervid PrP^{Sc} (Months 4-9)

1.4 Conduct SELEX for crude cervid PrP^{Sc} (Months 9-12)

1.5 Conduct negative SELEX for bovine recombinant normal prion ($rPrP^{C}$) (Months 12-15)

1.6 Assess enrichment of aptamer pool for target binding following round 6, 9, and 12 of SELEX (Months 9, 12, and 15)

Deliverable: Reduced aptamer pools selected for sensitive and specific recognition of PrP^{Sc}

Objective 2 Sequence selected aptamers species and down-select for aptamer beacon engineering (Months 15-24)

Tasks 2.1 Clone reduced aptamer pools from Objective 1.5 (Months 15-18)
2.2 Sequence aptamer clones (Months 18-21)
2.3 Assess sequence homologies from clones for family relationships (Month 21)
2.4 Determination of sensitivity and specificity of aptamers representative of aptamer families from 2.3 for CWD prion (PrP^{sc}) (Months 21-24)
2.5 Ranking of sensitivity and specificity of aptamer families for CWD prion (PrP^{sc}) (Month 24)

Deliverable: Aptamer sequences sensitive and specific recognition of PrP^{sc}

Progress on Objectives

1.1 Develop protocol for peptide library SELEX (Months 1-3)

The initial SELEX targeting strategy (Strategy#1) was modified based on a paper by Paramithiotis et al. (2003) describing a differential epitope that enabled antibodies generated against this epitope to discriminate the normal and misfolded forms of bovine PrP. This epitope is a tandem YYR motif which is common to all species of PrP is located at residues 152-154 and 165-167 for the elk prion protein.

1.2 Conduct SELEX with peptide library array for deer prion (PrP_{91-245}) (Months 3-9)
Y-Y-R Peptide as Aptamer Target

A tripepetide Y-Y-R was used as a target to select specific aptamers. This epitope was used to generate PrP^{SC} specific antibodies by, and was immobilized onto derivatized cellulose membranes using a commercially available kit (SPOTs™, Sigma Genosys). Technical problems with non-specific absorption of the aptamer pool to the Y-Y-R functionalized cellulose limited the likelihood of a successful outcome for this approach. At about the same time, the specificity of the Y-Y-R epitope for PrP^{SC} was called into question (Gorochov and Deslys, 2004) due to the potential for many other tyrosine-arginine motifs to be present in diagnostic samples such as blood. Because of the problems of non-specific binding to cellulose and doubtful PrP^{SC}-specificity of the YYR epitope, we decided to alter our selection strategy.

1.3 Prepare and assess preparation of crude cervid PrP^{sc} (Months 4-9)

We have successfully optimized and performed two methods of preparing cervid PrP^{SC} that have been developed by others: 1) a precipitation with phosphotungstate (PTA precipitation) method and 2) two ultracentrifugation methods.

1.4 Conduct SELEX for crude cervid PrP^{sc} (Months 9-12)

The ideal method of target presentation for aptamer selection is a free, unbound target in solution. This allows optimal interaction of the aptamer with all potential binding interfaces without the potential steric hindrance introduced by an immobilization

strategy. It also obviates the need for negative selection against the immobilization media. The primary drawback to such a presentation strategy is recovery of binding species. One group approached this problem by using capillary electrophoresis to separate bound from unbound species and were able to select binding aptamers within four rounds of selection (Mendonsa and Bowser 2004). We have developed a novel electrophoretic separation strategy using dialysis membranes to trap a relatively large protein target within an electric field after interaction with a pool of aptamers. After a period of electrophoresis, the unbound oligonucleotides are driven out of the membrane, leaving bound species and the protein target within the membrane. Aliquots of this electrodialysed solution inside the dialysis membrane are then used for PCR amplification of binding species.

1.5 Conduct negative SELEX for bovine recombinant normal prion ($rPrP^{C}$) (Months 12-15)

Negative selection steps were performed against recPrP. This negative selection is unique among SELEX strategies in that it utilizes an actual negative target (PrP^{C}) and not an immobilization substrate. Since our targets were free in solution, we had no immobilization substrate.

1.6 Assess enrichment of aptamer pool for target binding following round 6, 9, and 12 of SELEX (Months 9, 12, and 15)

CWD PrP^{SC} as Aptamer Target

Strategy #2 was devised in an attempt to exclude the complication of an immobilization substrate serving as an alternative target for oligonucleotide binding. PrP^{CWD} and recombinant PrP (rPrP) were used as positive and negative targets respectively, aptamers were allowed to interact with the targets in binding buffer, and separation of binding from non-binding species was accomplished by electrophoretic migration across a selective dialysis membrane. A total of fifteen rounds of SELEX were performed using the electrodialysis strategy outlined in Table 1. We were unable to demonstrate loss of sequence complexity by alignment of recovered sequences after rounds 4 and 8 or to demonstrate any difference in pool binding after rounds 4 and 8 to either form of PrP as measured by dot blotting or pull-down assay using ^{33}P labeled aptamers (data not shown). Due to the lack of binding and little loss of sequence complexity after rounds 4 and 8, we suspected that the current strength might not be high enough to thoroughly drive non-binding aptamers out of the dialysis membrane, and this was confirmed using ^{33}P oligonucleotides without a target in the dialysis membrane, but increasing the current to 70 mA improved partitioning. We subsequently performed an additional 2 rounds of negative selection and 5 rounds of positive selection using a 70 mA current.

Epitope Targeted Aptamer

Strategy #3 utilized motif-grafted antibodies obtained from Dr. R. Anthony Williamson at the Scripps Research Institute, La Jolla California as selection targets. These antibodies are engineered to contain polypeptides corresponding to residues 89-

112 or 136-158 of the mouse prion protein (residues 94-115 and 140-162 of elk prion protein) in the complementarity-determining region 3 of the heavy chain of a monoclonal mouse IgG specific for the envelope glycoprotein of HIV-1 (Solforosi et. al., 2004). These regions on PrP have been shown to be critical for the heterodimeric association of PrP^{C} and PrP^{SC} and are thought to represent differentially exposed epitopes on the two isoforms of the protein. Recent evidence suggests that IgG 89-112 specifically recognizes PrP^{SC} and further testing of this is ongoing (Moroncini et. al., 2006). In an attempt to direct selection of aptamers that specifically bind to PrP^{CWD}, we performed SELEX using motif grafted antibodies as targets for selection as described in materials and methods. As described by Moroncini et al. (2004), these antibodies were constructed by replacing the coding sequence of the extended chain complementarity-determining region 3 of monoclonal antibody b12 (a recombinant IgG developed against human immunodeficiency virus-1) with peptide sequences representing amino acid residues 90-113 (termed MAbA in this study) and 137-159 (termed MAbB in this study) of the elk prion protein sequence. These sequences contain residues thought to be important for the interaction between PrP^{C} and PrP^{SC} as evidenced by inhibition of prion propagation by Fab fragments reacting to regions in this region, antibodies recognizing this segment, and by synthetic PrP peptides spanning this region. We reasoned that since these regions are important for interaction between the two isoforms of PrP, epitopes within these regions might be differentially solvent exposed and thus aptamers that bind to these regions might differentially bind to one isoform or the other. Table 3 summarizes the rounds and targets used in this strategy. In an attempt to reduce non-specific binding to either the immunoglobulin backbone or the immobilization substrate, we performed negative selection with immobilized antibody b12.

Objective 2 Sequence selected aptamers species and down-select for aptamer beacon engineering (Months 15-24)

2.1 Clone reduced aptamer pools from Objective 1.5 (Months 15-18)
2.2 Sequence aptamer clones (Months 18-21)
2.3 Assess sequence homologies from clones for family relationships (Month 21)
2.4 Determination of sensitivity and specificity of aptamers representative of aptamer families from 2.3 for CWD prion (PrP^{sc}) (Months 21-24)
2.5 Ranking of sensitivity and specificity of aptamer families for CWD prion (PrP^{sc}) (Month 24)
2.6

Aptamers selected against CWD PrP^{SC} as Target

To assess the progress of enrichment for binding species, we cloned and sequenced aliquots of the pools at rounds four, eight, and fifteen. Enrichment is reflected by a loss of sequence diversity as the pool of random oligonucleotides is selected for binding to a particular target. The sequences of aptamer pools were analyzed by alignment and phylodendritic tree construction using Align X (Vector NTI Advance 10 software). The overall tree for 20 sequences from round 15 of electrodialysis SELEX is shown at Figure 1, and shows that the sequences fall into three broad families. This demonstrates that some enrichment has occurred, as one would expect that completely non-related sequences would show a much more diverse

dendrogram. Additionally, the alignment within these three families shows that significant consensus was conserved in families 1 and 3 (figures 2 and 3), although no sequences were identical.

We compared the sequences of our prion aptamers to those selected by other investigators (Table 2) and found that aptamer 15-8 was 55.6% identical to aptamer 60-1 selected by Sekiya et al. (2006) and 48.7% identical to aptamer M3 selected by Proske et al. (2002). Aptamer 15-7 also had strong identity with previously published aptamers, showing 28.6% identity with aptamer SAF-131 published by Rhie et al. (2003).

We proceeded to select candidate aptamers based upon the phylodendritic relationship and alignment evidence demonstrated. Aptamer 15-7 and 15-8 were selected for analysis of binding. The sequences of these aptamers are:

- 15-7: 5'- CGG GAA GGT GGA GCC GAG ACC GTT GTC ATC -3'
- 15-8: 5'- ACG GAG GTT CGG ATT AAT GCT GTG GTG CGC -3'.

In addition to aptamer 15-7 and 15-8, a randomly generated sequence (5'- GAG TCT TGG TGA CGT GCG TTG AGA TAC GCG -3' - scrambled aptamer) was also purchased for use as a control. All three aptamers were 5' end labeled as described and incubated with three different concentrations of target molecules. The bound and unbound fractions were determined by scintillation counting as described. Figures 4-6 show the comparisons of binding of different targets with each aptamer.

Statistical analysis of data was performed using JMP 6.0.0 from SAS and multiple means comparison for each aptamer at each concentration of target showed that binding of aptamer 15-8 was significantly different ($p < 0.05$) for both PrP^{CWD} and rPrP compared to binding of BSA (negative control) at all concentrations. The binding of aptamer 15-8 was not significantly different between PrP^{CWD} or rPrP at any concentration analyzed which could be due to binding to a commonly presented epitope on each target. The binding of aptamer 15-7 was significantly different ($p < 0.05$) for rPrP compared to PrP^{CWD} at concentrations of 250nM and 500nM, but was not significantly different when compared to BSA at any concentration. The binding of scrambled aptamer to all targets at all concentrations was not significantly different ($p <0.05$).

To compare the level of binding to a specific target (for example, the binding of aptamer 15-8 and 15-7 to CWD) would require a method to normalize the amounts of total bound activity in each experiment. We therefore included a control that would allow us to make such comparisons using *E. coli* single stranded DNA binding protein (SSB). This allowed calculation of percent specific binding using the equation:

% specific binding = (cpm bound to target – cpm bound to BSA / cpm bound to SSB – cpm bound to BSA) X 100

The results of these calculations are shown graphically in Figures 7 & 8 and show that the binding of aptamer 15-8 is two to four times higher for PrP^{CWD} than for SSB but the binding of aptamer 15-7 to CWD is less than the binding to SSB. Similarly,

the binding of aptaper 15-8 to rPrP was also higher than the binding to SSB whereas the binding of aptamer 15-7 was lower at all concentrations of target analyzed. As expected, the binding of scrambled aptamer to either CWD or rPrP was much lower than binding to SSB except at the lowest concentration of rPrP (100nM), which is probably a spurious result but might be a reflection of some studies which show that rPrP binds to nucleic acids.

Aptamers Selected against Motif Grafted Antibodies as Targets

We cloned and sequenced aliquots from each pool selected with MAbA or MAbB after round 12 and were unable to demonstrate loss of sequence diversity in either pool. Therefore, we performed an additional eight rounds of SELEX with each target and progressively increased the stringency of binding reactions by increasing the concentration of aptamer relative to target. This theoretically provides a level of competition such that the best binding species could displace species with lower binding affinity. As with the electrodialysis strategy, we compared the sequences recovered from round 20 for each target by phylodendritic tree construction and alignment using Align X.

Figure 9 shows the phylodendritic tree of sequences recovered after selection with MAb A. There are three major families, and the sequence of aptamer A15 is present in three copies, representing 15% of all sequences. The sequence of aptamer A11 is present in two copies, or 10% of all sequences. Additionally, aptamer A15 shows a 30.8% identity to the aptamer sequence published by Weiss et al. 1997 (Figure 10) and aptamer A11 shows strong identity to RM-312 (Mercey, R., et al. 2006) (Figure 11) and SAF-131 (Rhie, A., et al. 2003) (Figure 12). The phylodendritic tree of sequences recovered after selection with MAb B is shown at Figure 13. There are five major families with no dominant sequences identified, but aptamer B55 shares a series of motifs with aptamer A15 (Figure 14).

Aptamers A15, A11, and B55 were selected for analysis of binding. The sequence of these aptamers is:

- A15: 5' - TGC AGG TAT GGG GTA TCG CTC CTC CCC TAA - 3'
- A11: 5' – CTC CTA AAG CAC GGG GCC GTA AGC TGA TAG - 3'
- B55: 5'- GCT TCA CCG ACA GAG GTG AGG TAC GCT CAC - 3'

Binding of aptamers A15, A11, and B55 to targets were measured by scintillation counting of bound, radiolabeled aptamers to the targets similar to that described for electrodialysis SELEX. We measured the binding to a wider range of target concentrations (10nM to 500nM) and also included the target monoclonal antibody appropriate for each aptamer. The same scrambled aptamer as used for previously analysis of aptamers 15-7 and 15-8 was labeled and included as a control. Figures 15 through 18 show the comparisons of binding of different targets with each aptamer.

Statistical analysis of data was performed using JMP 6.0.0 from SAS and multiple means comparison for each aptamer at each concentration of target showed that binding of aptamer A15 was significantly different ($p < 0.05$) for PrP^{CWD} and MAbA compared to binding of BSA (negative control) at all concentrations except 100 nM (for PrP^{CWD} – see table 3-4). The binding of apatmer A15 to rPrP was not significantly different from the binding to BSA except at 25 nM and 50 nM. The binding of aptamer

A11 was significantly different between PrP^{CWD} and BSA at all concentrations above 33nM, and the binding of this aptamer to rPrP was significantly different from BSA at target concentrations above 250nM (table 3-5). Aptamer B55 was shown to bind to PrP^{CWD} at significantly higher levels than BSA at all target concentrations except 10 nM and 500nM and bound to rPrP at significant levels as compared to BSA (table 3-6) at concentrations of 250nM and 500nM.

As with the aptamers 15-7 and 15-8, we calculated the specific binding of each aptamer to PrP^{CWD} and to rPrP as a percentage of binding measured for each aptamer to SSB. The results of these calculations are shown graphically at figures 19 through 22 and show that the binding of aptamer A15 to PrP^{CWD} was 20 to 80% of that measured for SSB whereas the binding of this aptamer to rPrP was less than 40% of that measured for SSB at all concentrations. The binding of aptamer A11 to PrP^{CWD} and rPrP was approximately 20 to 80% of SSB, and the binding of aptamer B55 was generally 30 to 60% of SSB.

Objective 3 Engineer aptamer beacons and assess performance for recognition of CWD PrPsc (Months 25-36)

Tasks 3.1 Identification of target recognition sequences for aptamer beacon candidates (Months 25-28)

3.2 Synthesis and assessment of candidate aptamer beacons with peptide targets (Months 28-32)

3.3 Assessment of candidate aptamer beacon performance with crude PrPsc (Months 32-36)

Deliverable: Aptamer beacons sensitive and specific recognition of PrPsc

No work was accomplished on Objective 3 owning to the longer time and larger expense needed to accomplish Objectives 1 and 2.

Key Research Accomplishments

- Technical difficulties negated selection of aptamers against immobilized prion peptides as originally proposed
- Aptamers 15-7 and 15-8 selected against $rPrP^{C}$ and PrP^{CWD} binds both PrP^{CWD} and rPrP significantly greater than BSA (negative control), but these aptamers showed no selectivity between PrP^{CWD} or $rPrP^{C}$
- PrP^{CWD} binds greater amounts of aptamer 15-8 than *E coli* single-strand DNA binding protein suggesting high affinity binding of aptamer 15-8 for PrP^{CWD}
- Aptamer A15 selected against a elk prion motif thought to be important in mis-folding binds to PrP^{CWD} at significantly higher levels compared to BSA and the specific binding of this aptamer indicate that it may has some specificity for PrP^{CWD} over PrP^{C}
- These aptamers selected against $rPrP^{C}$ and PrP^{CWD} had sequence homology with those previously selected by others against prions from non-cervid species suggesting that particular shared prion motifs are exposed on prions

Reportable Outcomes

Presentations:

Blair JB, Clinkenbeard KD Selection of an Aptamer for Diagnosis of Chronic Wasting Disease. *National Prion Research Program Meeting* 2005, Washington, DC.

Jeff Blair, Development of a Novel Electrodialysis Method for SELEX Oklahoma State University Graduate College Research Symposium (02-24-2005)

Clinkenbeard KD, Jean Clarke J, Malayer JR, Hancock LF, Moon JH, Guo N, Timothy A. Snider TA, Dye R, Wang S. Aptamers for Detection of Biowarfare Agents. *Army Research Office Workshop for on the Chip Detection of Biological and Chemical Molecules.* 2004, Raleigh, NC.

Jeff Blair, Selection of an Aptamer to the Misfolded Prion Protein of Chronic Wasting Disease Oklahoma State University Graduate College Research Symposium (03-05-2004)

Jeff Blair, Kenneth Clinkenbeard, Aptamers as Diagnostic Tools for Transmissible Spongiform Encephalopathies, TSE in the Americas Conference, Ames, IA (10-07-2005)

Jeff Blair; Will Sims; Katherine I. O'Rourke[§]; Kenneth D. Clinkenbeard. Discovery Research to Select an Aptamer to the Mis-folded Prion Protein of Chronic Wasting Disease. Oklahoma State University CVM Phi Zeta Research Day (03-18-2004)

Blair JL, Clinkenbeard K, and O'Rourke K. Selection of an aptamer to the misfolded prion protein of chronic wasting disease. Oklahoma State University Food and Agricultural Products Research and Technology Center Symposium (04-19-2004)

Publications: Jeffrey L. Blair, APTAMER SELECTION AND CELL CULTURE MODEL DEVELOPMENT FOR DIAGNOSIS OF CHRONIC WASTING DISEASE OF CERVIDAE, PhD Dissertation, Oklahoma State University, Stillwater, OK, May 2007.

Graduate Student(s) Supported: Jeffrey Blair, DVM enrolled in Veterinary Biomedical Sciences PhD program

Funding Applied for Based on this Work Supported by this Award: Small Business Technology Transfer (STTR) Program, Proposal Number: A045-027-0237; Topic Number: A04-T027; Ruminant B-Lymphocyte Yellow Fluorescent Protein Aggregation Bioassay for Elk Chronic Wasting Disease

Employment applied for and/or received based on experience/training supported by this award:

Jeffrey Blair, DVM, PhD hired as Senior Veterinary Scientist by Meriel Animal Health in St Louis, MO.

Conclusions

The goal for this project was to select aptamers specific for PrP^{CWD} and to employ these aptamers as diagnostic probes for an antemortem diagnostic test for CWD. Three strategies were used to select aptamers specific for PrP^{CWD}. The original strategy using selection against prion peptides was scarped owing to technical difficulties. Selection using a novel electrodialysis SELEX method that we developed using $rPrP^{C}$ and PrP^{CWD} selected two aptamers (15-7 & 15-8) that binds to both

isoforms of PrP as shown by comparison to binding a negative control protein (BSA). Aptamer 15-8 also binds to PrP^{CWD} and rPrP at levels higher than SSB as shown by percent specific binding. The apparent lack of significant differential binding to PrP^{CWD} as compared to PrP^{C} is similar to the findings of other investigators, indicating that the full length prion protein in either isoform may be a poor target for selection of conformation-specific aptamers. Successful selection of PrP^{CWD}-specific aptamers may depend upon presentation of differentially exposed epitopes as selection targets in the absence of confounding common regions of the two isoforms of prion protein that may bind nucleic acids with high affinity.

Strategy 3 used motif grafted monoclonal antibodies as targets displaying elk PrP motifs. Three aptamers were selected. Aptamer A15 binds to PrP^{CWD} at significantly higher levels compared to BSA and the specific binding of this aptamer indicate that it may has some specificity for PrP^{CWD} over PrP^{C}. The binding of aptamers A11 and B55 to PrP^{CWD} and rPrP were not as consistently different from BSA and the specific binding calculated using SSB were also not consistently different.

In summary, we have identified a total of five potential aptamers that can bind to prion protein. Of these, aptamer A15 binds to PrP^{CWD} at higher levels than rPrP, and aptamer 15-8 binds to both PrP^{CWD} and rPrP at higher affinity levels than SSB. Studies of the kinetics of the binding of each of these aptamers are warranted to identify and further clarify the potential of any of these aptamers as specific probes for PrP^{CWD}. Although we attempted several strategies aimed at selecting a specific aptamer that could recognize PrP^{CWD} and differentiate it from PrP^{C}, we were only moderately successful. Selection of a specific aptamer that can bind to the abnormal, disease associated isoform of prion protein has proven difficult for many investigators. There have been at least five published studies describing the selection of aptamers to prion protein and only two of these claim to have selected aptamers that preferentially bind PrP^{SC}, but neither of these has been developed into a diagnostic reagent. This may indicate that selection of a specific aptamer for the disease associated form of prion protein will require alternative approaches of selection not yet considered. The two isoforms of prion protein should theoretically be distinguishable by aptamers, as aptamers have been shown to distinguish even different enantiomers of the same molecule. Although the exact tertiary structure of PrP^{SC} is unknown, it is known that the two isoforms differ in secondary and tertiary structure which should present at least one differential target for selection. Therefore, the difficulty in selecting a specific aptamer for PrP^{SC} (or PrP^{CWD}) is unlikely due to the inability of aptamers to resolve differences from PrP^{C}. Potential factors that could account for the lack of selection of a specific aptamer include nonspecific nucleic acid binding properties of PrP and insolubility of PrP^{SC} in selection buffers. The field of aptamer selection is growing increasingly sophisticated and selection of a specific aptamer for PrP^{SC} may yet be accomplished in future studies

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Appendix

Table 1. Outline of electrodialysis SELEX. Negative selection was performed at rounds 5-6 and rounds 9-10 in an attempt to remove oligonucleotides that bind to PrP^{C}.

Round	Incubation Time	Target	Moles Target:Moles Aptamer Pool	Selection Strategy	Assessment
1-4	1 hour	PrP^{CWD}	1:1	Positive	Binding assay and sequencing
5-6	1 hour	recPrP	1:1	Negative	
7-8	1 hour	PrP^{CWD}	1:10	Positive	Binding assay and sequencing
9-10	1 hour	recPrP	1:1	Negative	
11-15	30 minutes	PrP^{CWD}	1:1	Positive	Binding assay and sequencing

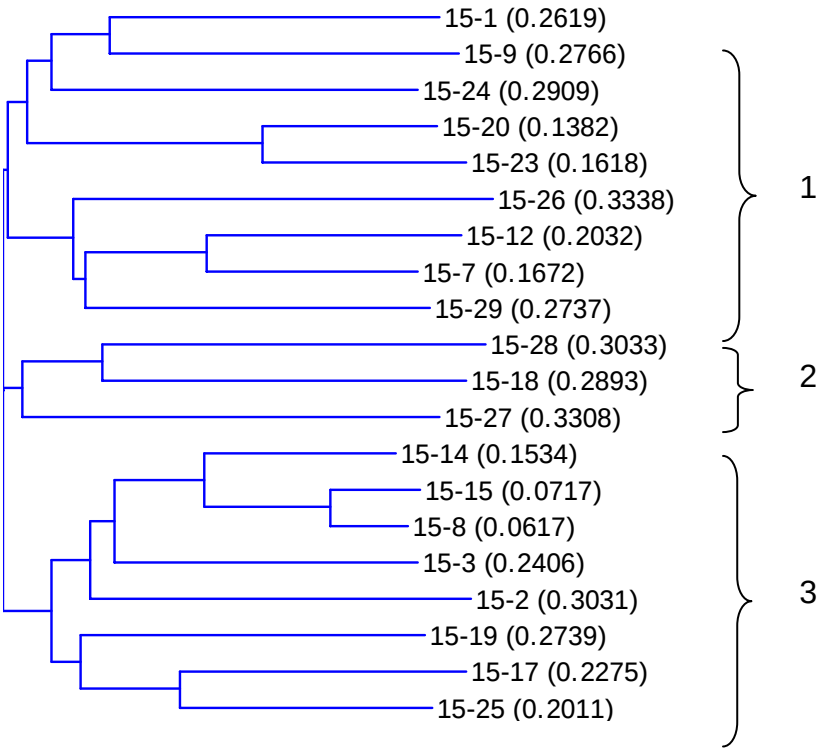
Table 2. Summary of published studies to select aptamers to prion protein.

Author/Date	RNA/DNA	Interaction site	PrPSC specific?
Weiss 1997	RNA	23-52	No
Proske 2002	RNA	90-124	No
Rhie 2003	RNA	23-110 >110	No Yes
Sekiya 2006	RNA	23-108	Possible
Takemura 2006	DNA	<90	No

Table 3. Outline of targeted epitope SELEX. Negative selection was performed in rounds 1, 2, 13, and 14 to remove aptamers that bind to the gel substrate or areas of antibody b12 outside the motifs grafted into the complementarity determining region.

Round	**Incubation Time**	**Target**	**Moles Target:Moles Aptamer Pool**	**Selection Strategy**	**Assessment**
1-2	1 hour	b12	1:1	Negative	
3-12	1 hour	MAb A MAb B	1:1	Positive	Sequencing
13-14	1 hour	b12	1:1	Negative	
15-18	1 hour	MAb A MAb B	1:2	Positive	
19-20	1 hour	MAb A MAb B	1:10	Positive	Sequencing

Figure 1. Phylodendritic tree showing relationships of 20 aptamer sequences after 15 rounds of selection by electrodialysis SELEX.




```
                  (1) 1         10        20        30        47
Aptamer 15-1      (1) -------ACGTGTAAACCATTGTAACTAGCTGCTG--TGTC------
Aptamer 15-12     (1) ---------GGGAAG-GGTATCGACAC-ACCACCA-ACGTCC-----
Aptamer 15-20     (1) -----ATACATCTAG-----TCTGCATCCTTAC---TTGTAGA----
Aptamer 15-23     (1) -----ATGCATGCAG-C---TCTACNTGTTTAC---TTGCTGAG---
Aptamer 15-24     (1) --------CTTGGACCAAATTATAACCCGCTCCGCCT---------
Aptamer 15-26     (1) GTAACCCGCACTTAGCAGCGTGAACACCTC----------------
Aptamer 15-29     (1) ----------CTCCG-C---TCGACCC-AGTACCGCTTGT--ACCGA
Aptamer 15-7      (1) --------CGGGAAG-G---TGGAGCCGAG-ACCG-TTGTC-ATC--
Aptamer 15-9      (1) --ATGACCAATCTAG-CGATAGTAGTCCGCTAC-------------
Consensus         (1)          C TG AG     T TAC C   TAC    TGT
```

Figure 2. Multiple sequence alignment of family 1 from sequences of round 15 electrodialysis SELEX.

```
                (1) 1          10         20         30        47
Aptamer 15-14   (1) ----------CTGA--TCTAGG---CTGC----------------
Aptamer 15-19   (1) --TGGTGCTGGCTGAGGTATAAG---CTGTC-T-G--TA--------
Aptamer 15-15   (1) ACGGATGCT--CGGA--TCAATG---CTGTGGT-G--CTC-------
Aptamer 15-8    (1) ACGGAGGTT--CGGA--TTAATG---CTGTGGT-G--CGC-------
Aptamer 15-17   (1) -------CACTCAGACAGTGGGT---GGGTGATCGGCTAG-------
Aptamer 15-3    (1) ----------AGGACGGATGTGATGCAACCTTCGGTCAAC------
Aptamer 15-2    (1) ----------GATAGNTCTGGTACTGGGAT-GGATACCTCG---
Aptamer 15-25   (1) ----------AAGNTGGCTACG---CTTTGATAG--TACGTGGTAT
Consensus       (1)           C GA   T TATG    CTGTG T G   TAC
```

Figure 3. Multiple sequence alignment of family 3 from sequences of round 15 electrodialysis SELEX

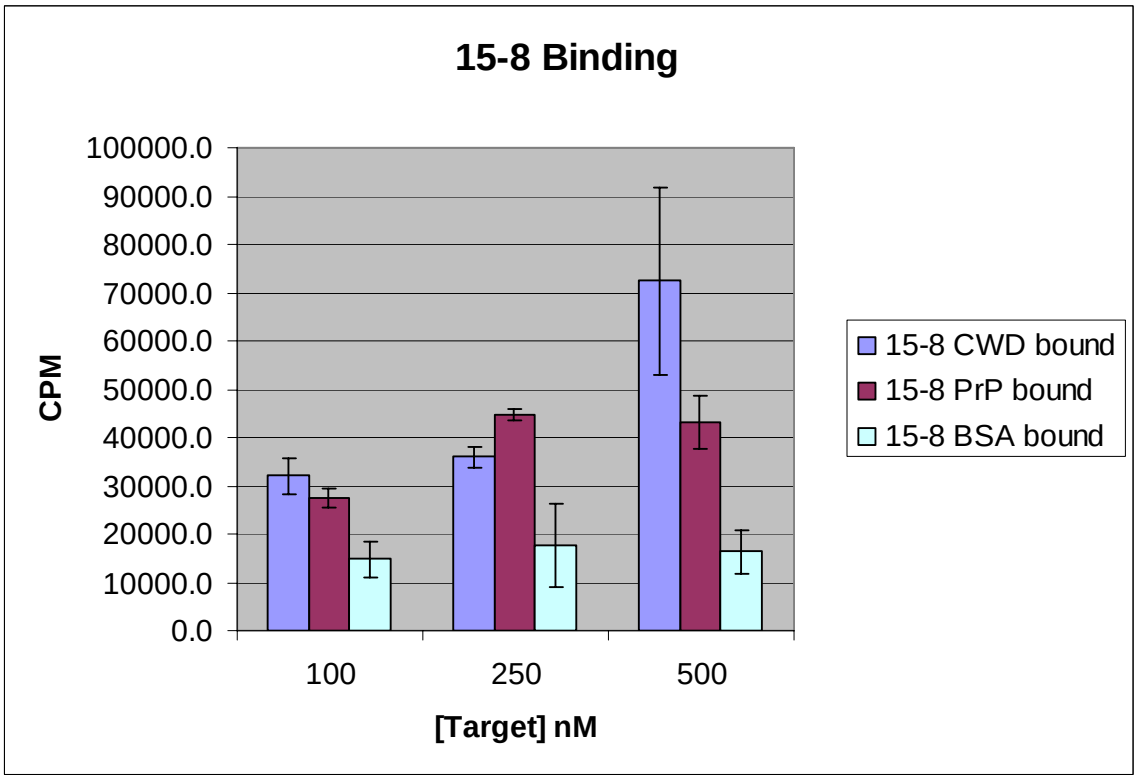


Figure 4. Binding of radiolabeled aptamer 15-8 to different concentrations of target molecules as measured by total scintilation counts pulled down. 100,000 cpm of labeled aptamer was incubated with indicated concentration of target for one hour in HMKN buffer. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting. Binding of 15-8 to CWD and rPrP was significantly different from BSA as measured by Tukey-Kramer HSD analysis ($p < 0.05$) at all three concentrations of target, as indicated by the asterisks.

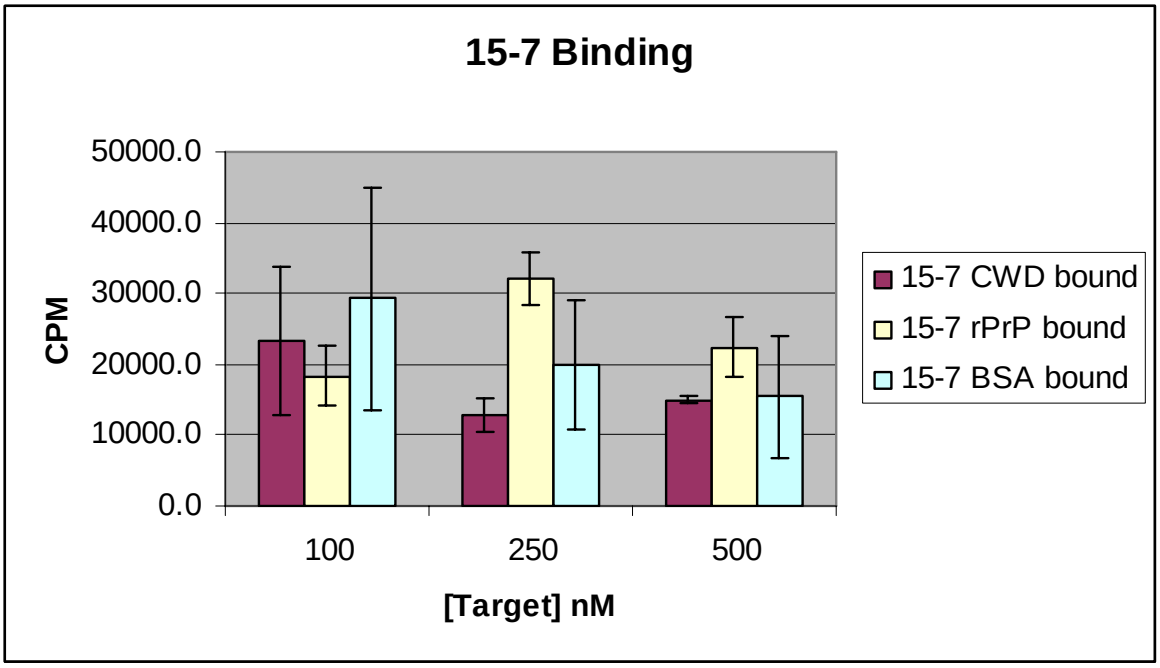


Figure 5. Binding of radiolabeled aptamer 15-7 to different concentrations of target molecules as measured by total scintilation counts pulled down. 100,000 cpm of labeled aptamer was incubated with indicated concentration of target for one hour in HMKN buffer. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting. Binding of 15-7 to CWD or rPrP was not signicantly different from BSA as measured by Tukey-Kramer HSD analysis ($p < 0.05$), but 15-7 binding to rPrP was significantly different than binding to CWD at concentrations of 250 nM and 500 nM.

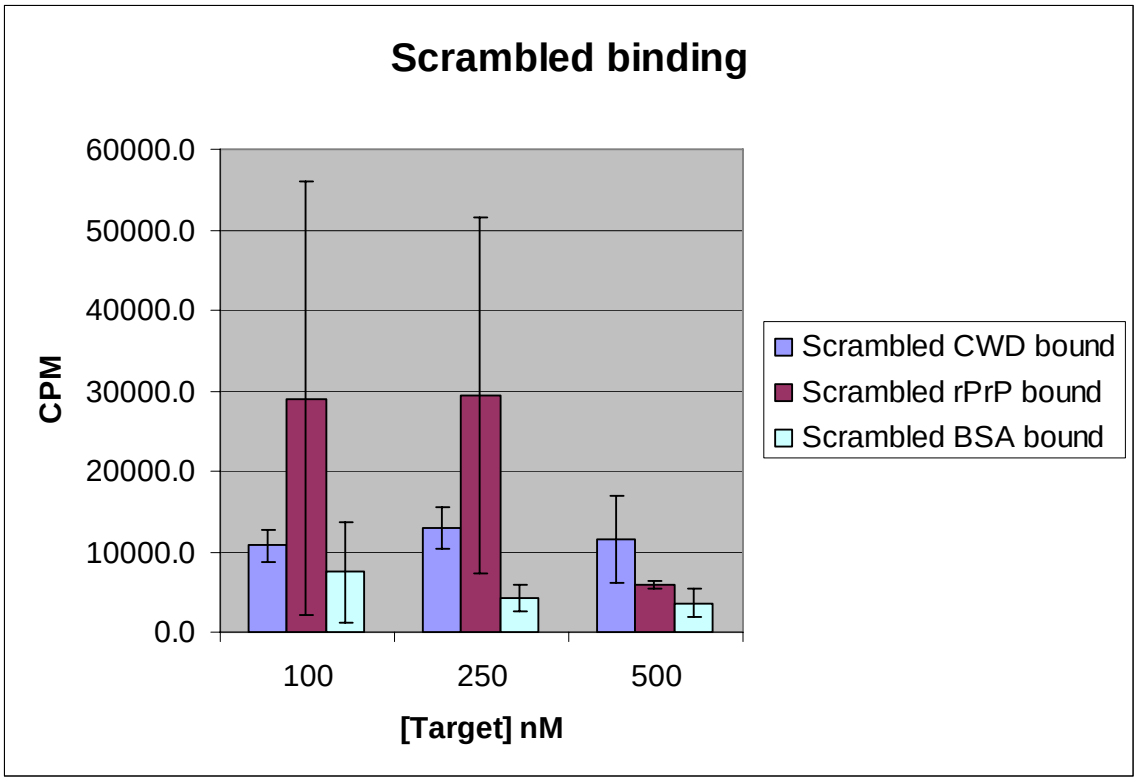


Figure 6. Binding of radiolabeled scrambled aptamer to different concentrations of target molecules as measured by total scintilation counts pulled down. 100,000 cpm of labeled aptamer was incubated with indicated concentration of target for one hour in HMKN buffer. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting. Binding of scrambled aptamer was not signicantly different as measured by Tukey-Kramer HSD analysis ($p < 0.05$).

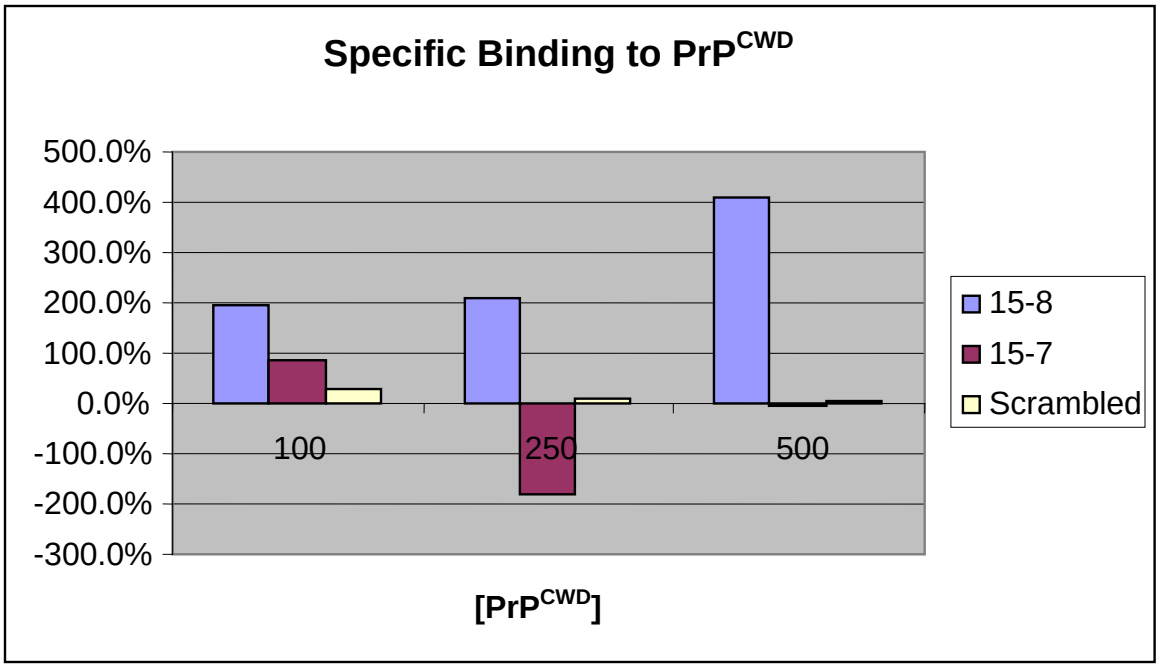


Figure 7. Specific binding of selected aptamers to PrP^{CWD} as percent of bound to SSB minus bound to BSA. Radiolabeled aptamers were incubated with the indicated concentrations of PrP^{CWD}, BSA, and SSB for one hour in HMKN. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting. Specific binding was calculated by subtracting the counts bound to BSA from the counts bound to PrP^{CWD} and SSB, then dividing the difference of PrP^{CWD} by the difference of SSB.

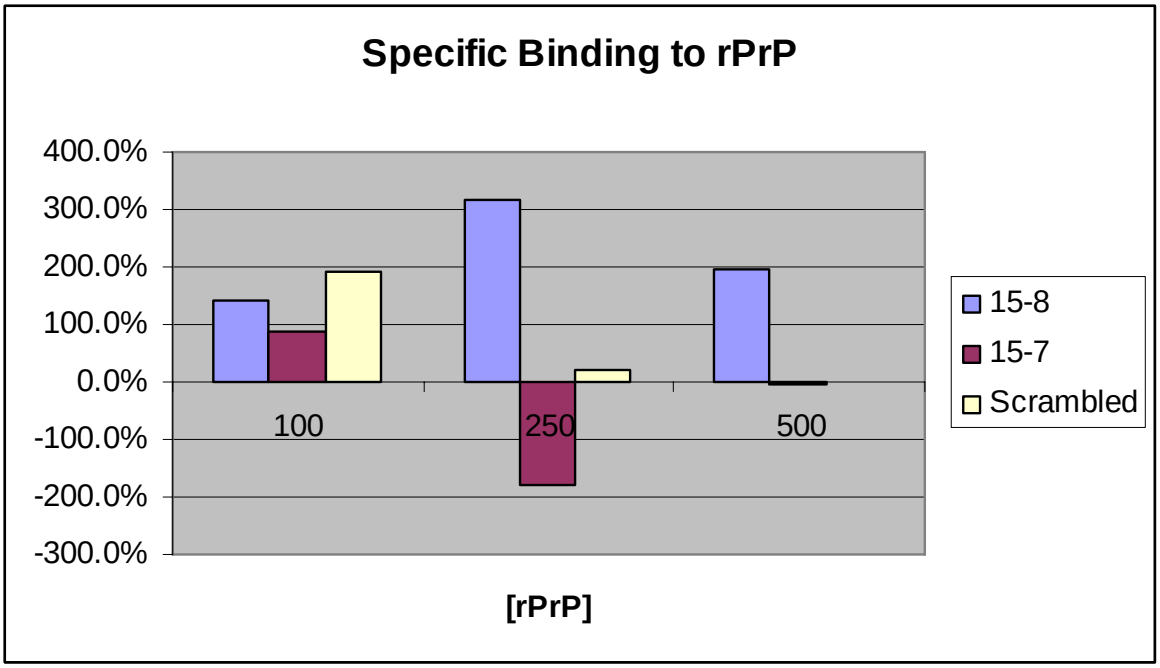


Figure 8. Specific binding of selected aptamers to rPrP as percent of bound to SSB minus bound to BSA. Radiolabeled aptamers were incubated with the indicated concentrations of rPrP, BSA, and SSB for one hour in HMKN. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting. Specific binding was calculated by subtracting the counts bound to BSA from the counts bound to rPrP and SSB, then dividing the difference of rPrP by the difference of SSB.

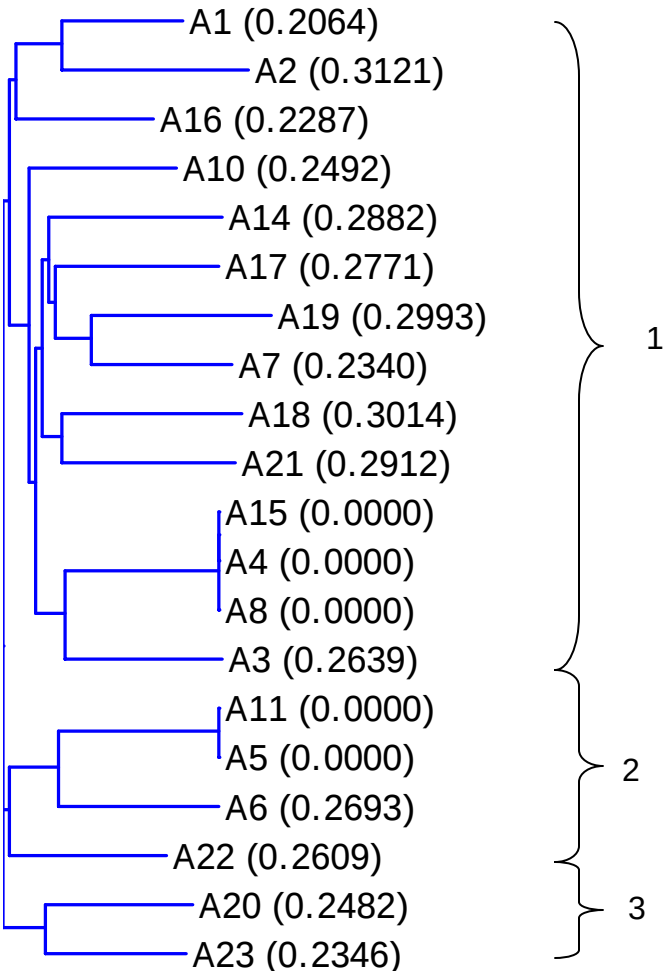


Figure 9. Phylodendritic tree showing relationships of 20 aptamer sequences after 20 rounds of selection by targeted epitope SELEX with MAbA. Three major families are present as indicated by the brackets. The species represented by A8 comprising 15% of seequences recovered and A11 representing 10% of sequences recovered.

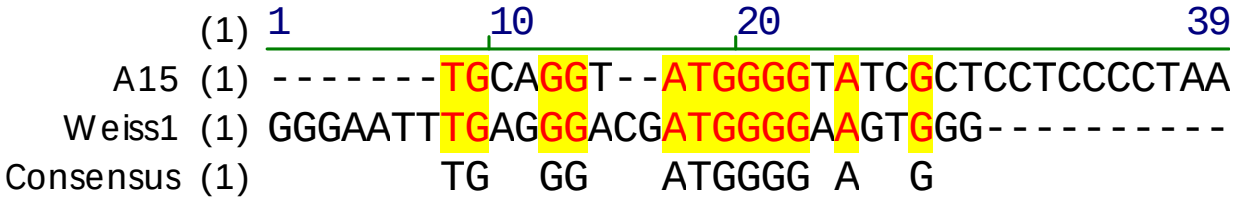


Figure 10. Individual sequence alignments of aptamers A15 and the aptamer selected by Weiss et. al showing 30.8% identity.

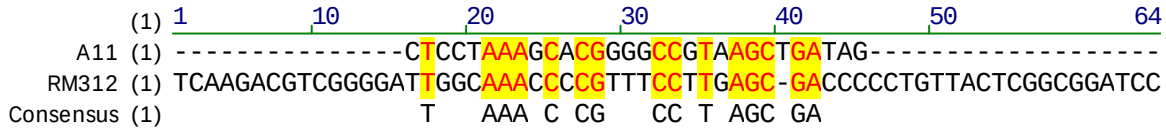


Figure 11. Individual sequence alignments of aptamers A11 and RM312 showing 23.4% identity.

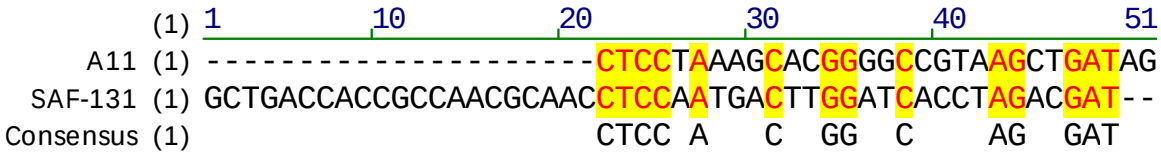


Figure 12. Individual sequence alignments of aptamers A11 and SAF-131 showing 27.5% identity

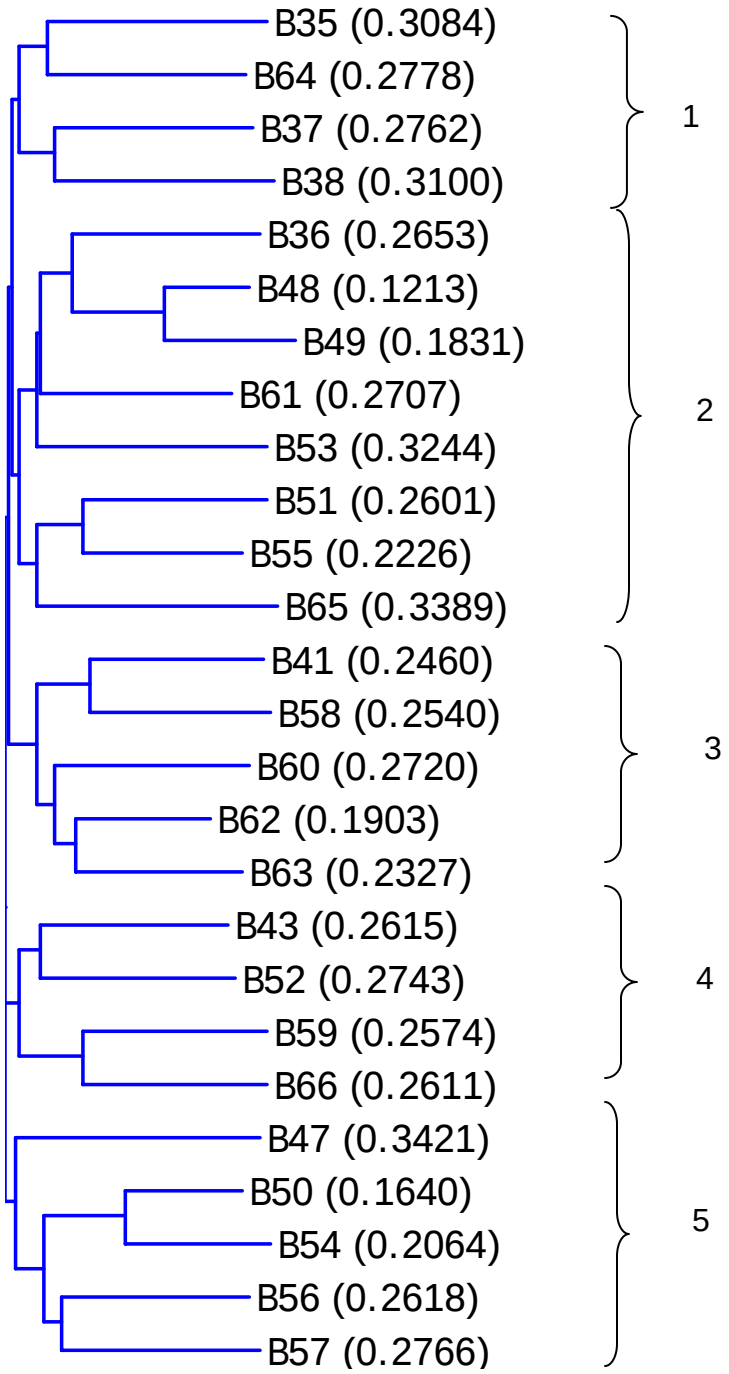


Figure 13. Phylodendritic tree showing relationships of 26 aptamer sequences after 20 rounds of selection by targeted epitope SELEX with MAbB. Five major families are indicated by brackets.

```
          (1) 1          10         20          38
A15       (1) --------TGCAGGTATGGGGTATCGCTCCTCCCCTAA
B55       (1) GCTTCACCGACAGAGGTGAGGTA-CGCTCAC-------
Consensus (1)          CAG    TG GGTA CGCTC
```

Figure 14. Individual sequence alignments of aptamers A15 and B55 showing the series of common motifs identified.

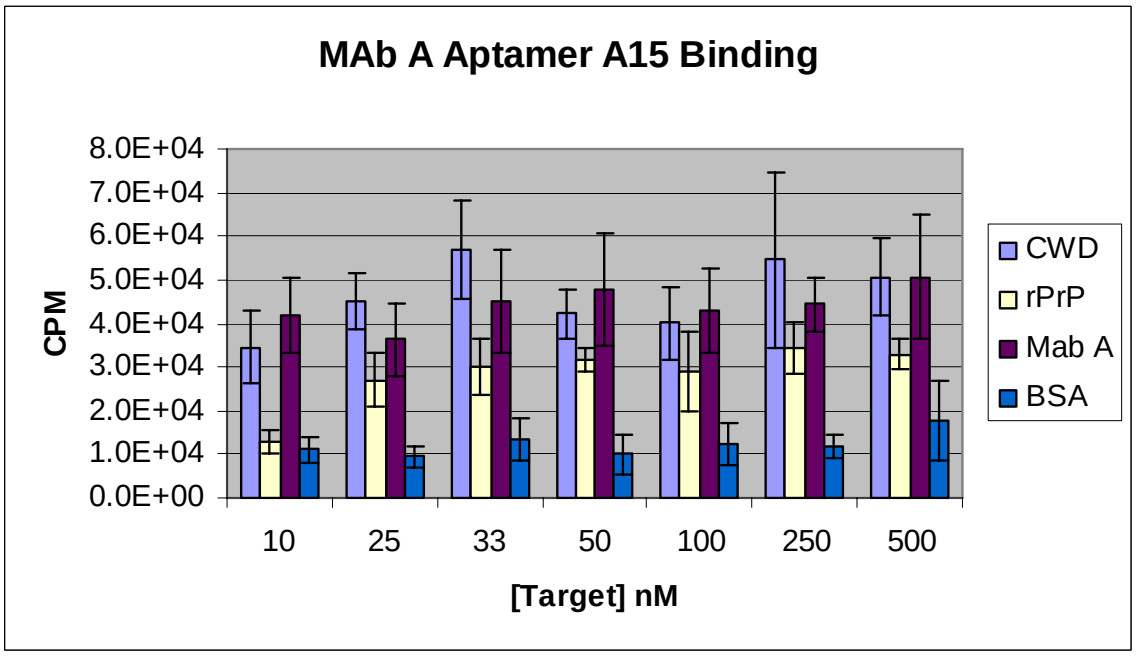


Figure 15. Binding of radiolabled MAb A Aptamer A15 to different concentrations of target molecules as measured by total scintillation counts pulled down. 100,000 cpm of labled aptamer was incubated with indicated concentration of target for one hour in HMKN buffer. The protein targets and bound aptamers were separated from unbound aptamers by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting.

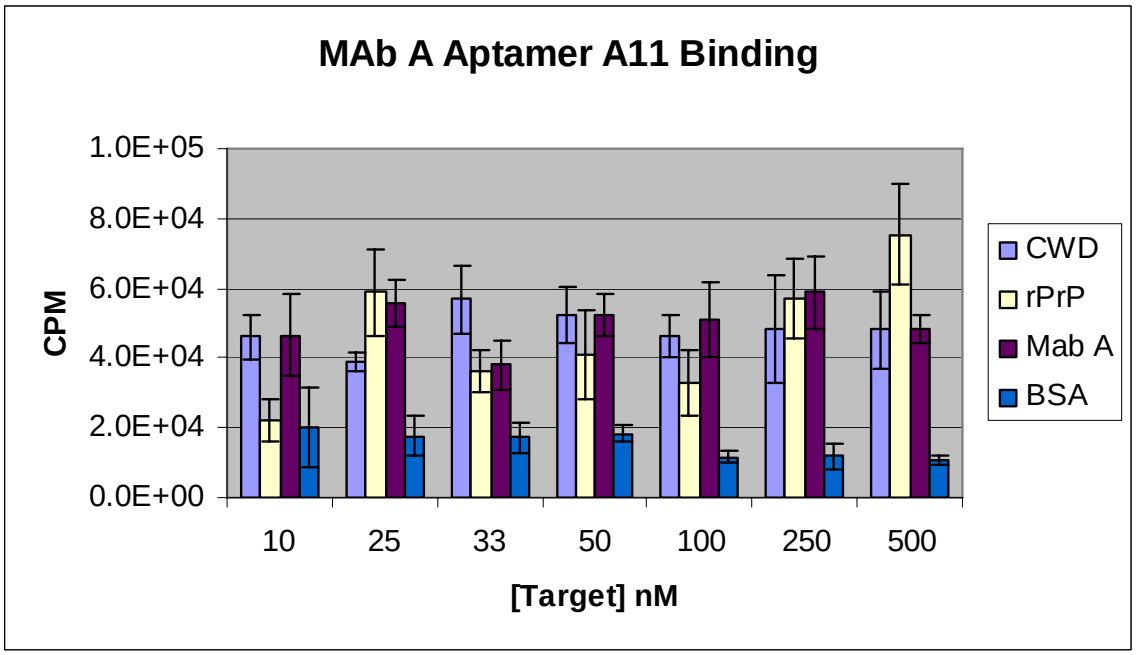


Figure 16. Binding of radiolabled MAb A Aptamer A11 to different concentrations of target molecules as measured by total scintillation counts pulled down. 100,000 cpm of labled aptamer was incubated with indicated concentration of target for one hour in HMKN buffer. The protein targets and bound aptamers were separated from unbound aptamers by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting.

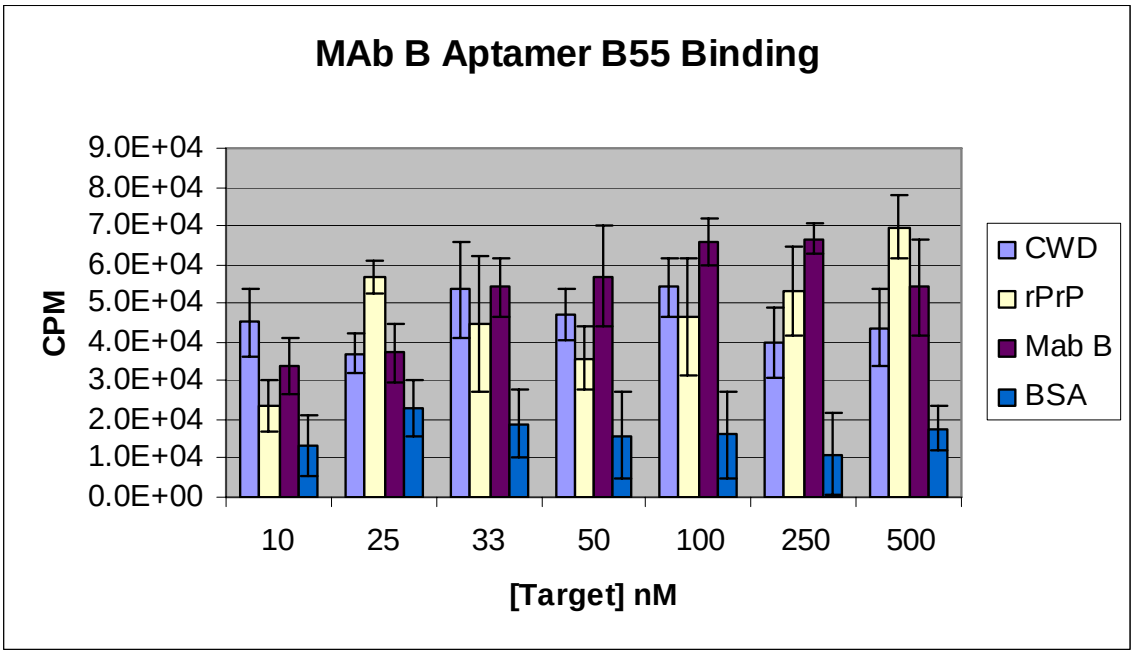


Figure 17. Binding of radiolabled MAb b Aptamer B55 to different concentrations of target molecules as measured by total scintillation counts pulled down. 100,000 cpm of labled aptamer was incubated with indicated concentration of target for one hour in HMKN buffer. The protein targets and bound aptamers were separated from unbound aptamers by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting.

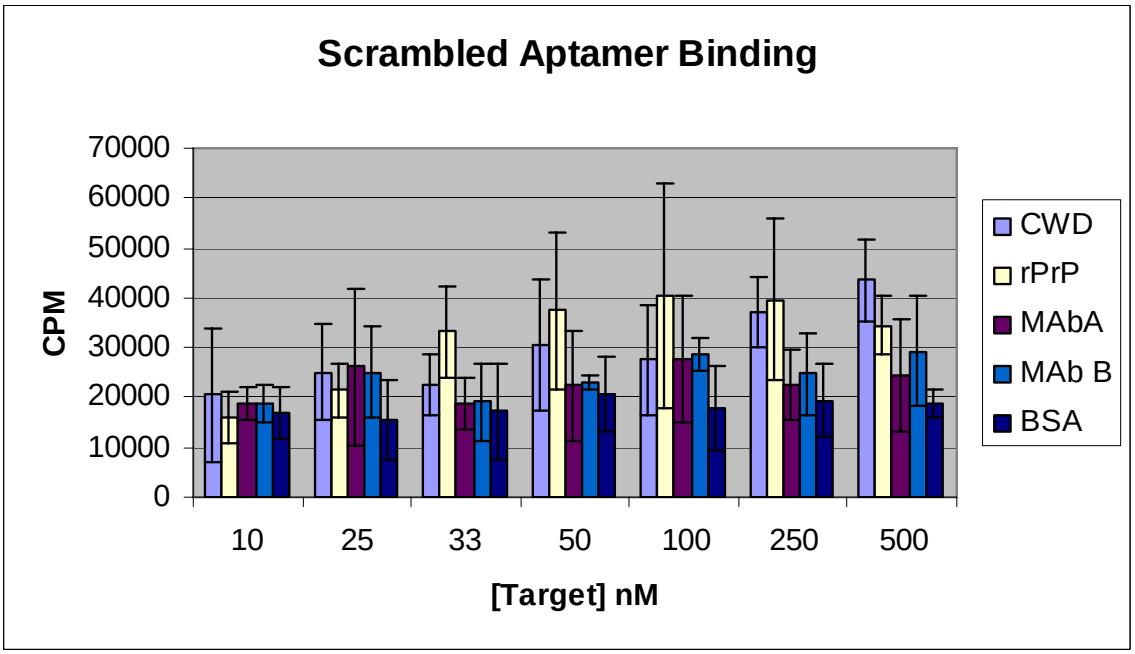


Figure 18. Binding of radiolabeled scrambled aptamer to different concentrations of target molecules as measured by total scintilation counts pulled down. 100,000 cpm of labeled aptamer was incubated with indicated concentration of target for one hour in HMKN buffer. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting.

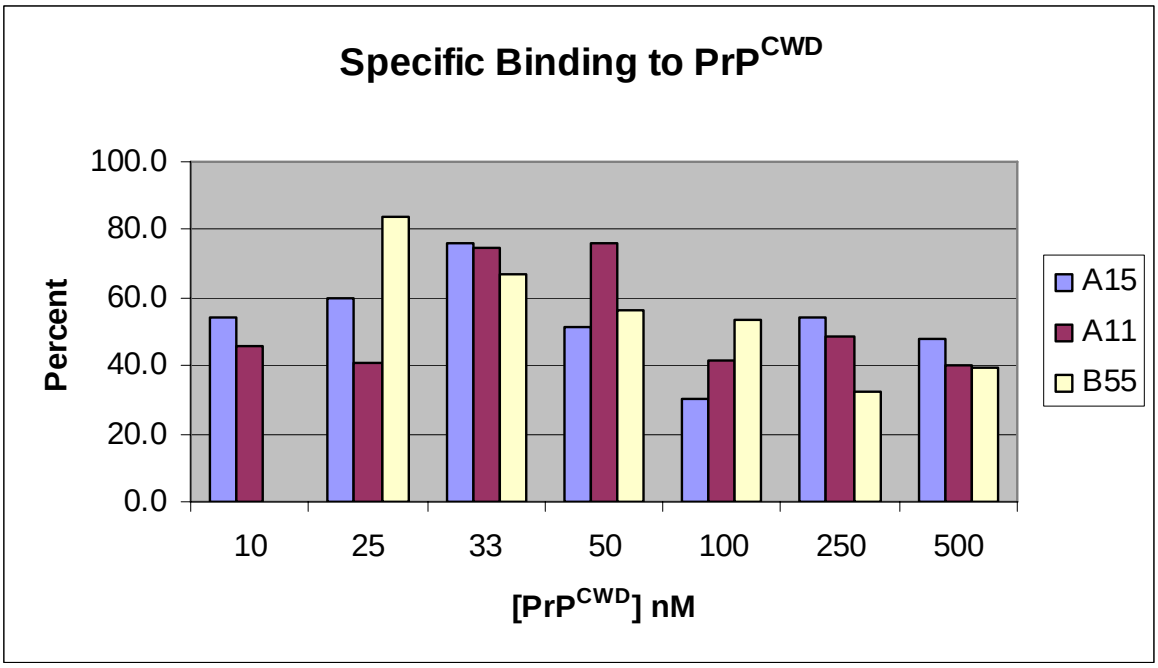


Figure 19. Specific binding of selected aptamers to PrP^{CWD} as percent of bound to SSB minus bound to BSA. Radiolabeled aptamers were incubated with the indicated concentrations of PrP^{CWD}, BSA, and SSB for one hour in HMKN. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting. Specific binding was calculated by subtracting the counts bound to BSA from the counts bound to PrP^{CWD} and SSB, then dividing the difference of PrP^{CWD} by the difference of SSB.

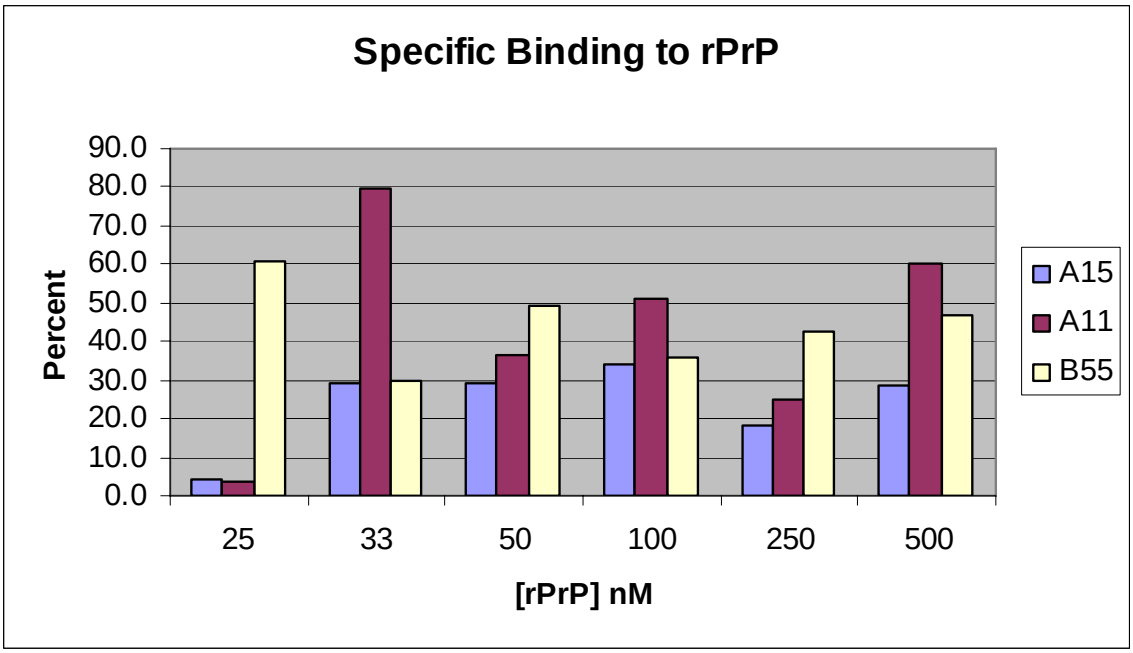


Figure 20. Specific binding of selected aptamers to rPrP as percent of bound to SSB minus bound to BSA. Radiolabeled aptamers were incubated with the indicated concentrations of rPrP, BSA, and SSB for one hour in HMKN. The protein targets and bound oligonucleotides were separated from unbound oligonucleotides by the addition of Strataclean resin. The resin was washed twice with HMKN and placed in scintillation fluid for scintillation counting. Specific binding was calculated by subtracting the counts bound to BSA from the counts bound to rPrP and SSB, then dividing the difference of rPrP by the difference of SSB.

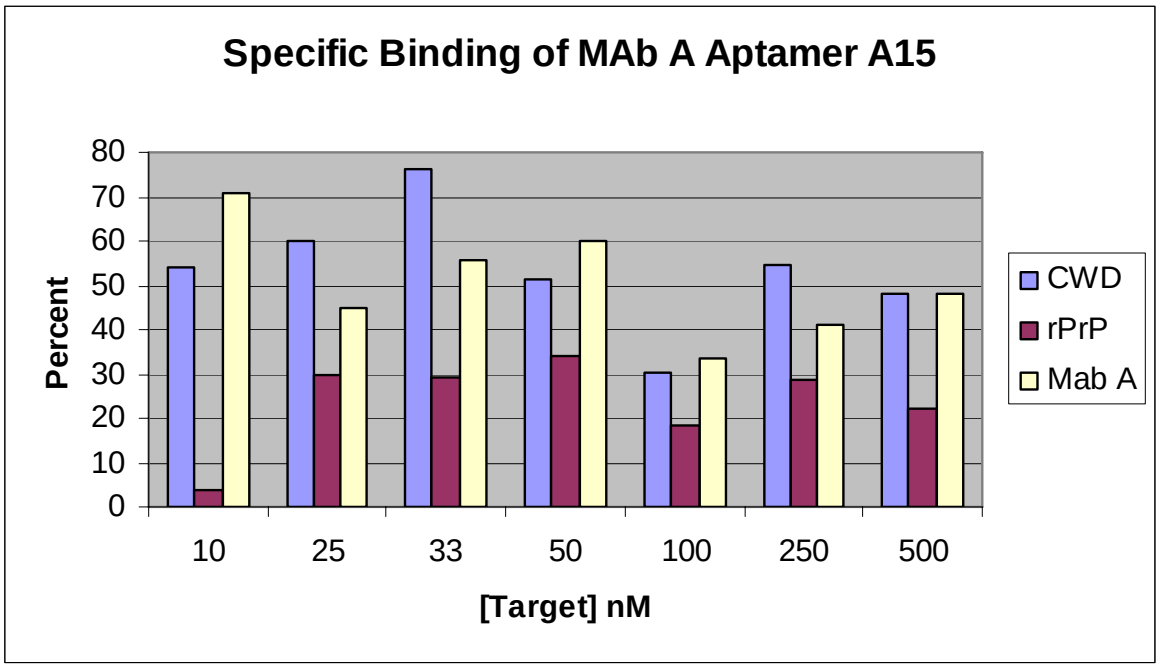


Figure 21. Specific binding of aptamerA15 to PrP^{CWD}, rPrP, and MAbA as percent of bound to SSB minus bound to BSA.

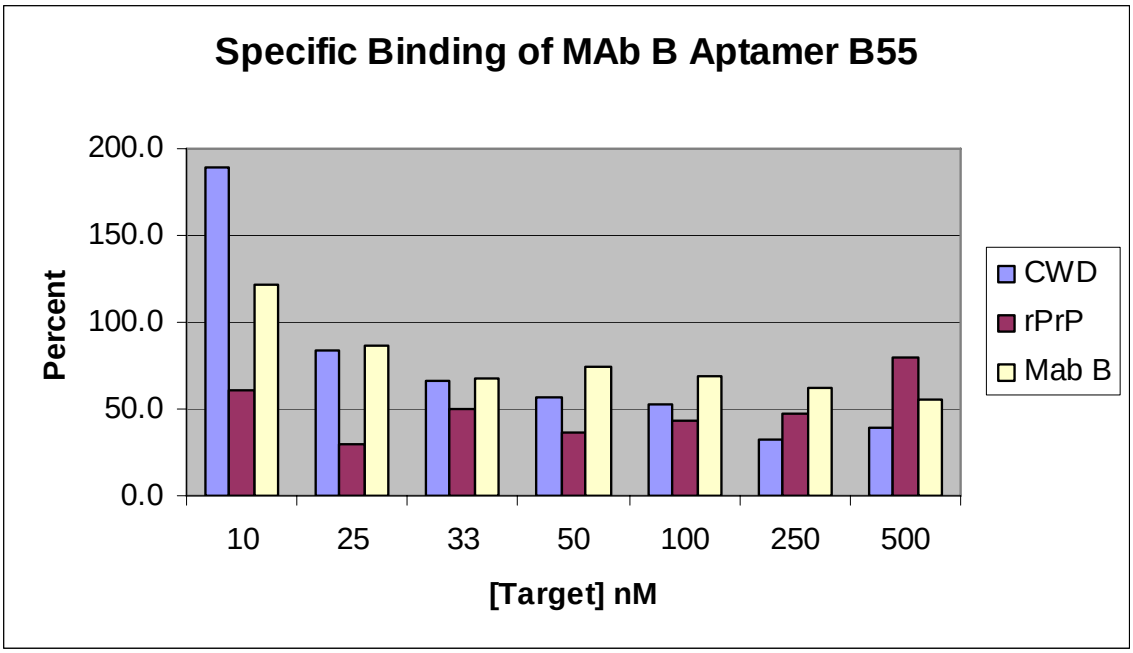


Figure 22. Specific binding of aptamerB55 to PrPCWD, rPrP, and MAbB as percent of bound to SSB minus bound to BSA.